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VanB-Type *Enterococcus faecium* Clinical Isolate Successively Inducibly Resistant to, Dependent on, and Constitutively Resistant to Vancomycin[▽]

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Three *Enterococcus faecium* strains isolated successively from the same patient, vancomycin-resistant strain BM4659, vancomycin-dependent strain BM4660, and vancomycin-revertant strain BM4661, were indistinguishable by pulsed-field gel electrophoresis and harbored plasmid pIP846, which confers VanB-type resistance. The vancomycin dependence of strain BM4660 was due to mutation $P_{175}L$, which suppressed the activity of the host Ddl D-Ala:D-Ala ligase. Reversion to resistance in strain BM4661 was due to a G-to-C transversion in the transcription terminator of the *vanRS_B* operon that lowered the free energy of pairing from −13.08 to −6.65 kcal/mol, leading to low-level constitutive expression of the resistance genes from the P_{RB} promoter, as indicated by analysis of peptidoglycan precursors and of $VanX_B$ D,D-dipeptidase activity. Transcription of the resistance genes, studied by Northern hybridization and reverse transcription, initiated from the P_{YB} resistance promoter, was inducible in strains BM4659 and BM4660, whereas it started from the P_{RB} regulatory promoter in strain BM4661, where it was superinducible. Strain BM4661 provides the first example of reversion to vancomycin resistance of a VanB-type dependent strain not due to a compensatory mutation in the *ddl* or $vanS_B$ gene. Instead, a mutation in the transcription terminator of the regulatory genes resulted in transcriptional readthrough of the resistance genes from the P_{RB} promoter in the absence of vancomycin.

Glycopeptide antibiotics, vancomycin and teicoplanin, are widely used for the treatment of severe infections due to gram-positive bacteria. They inhibit peptidoglycan synthesis by binding to the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) of accessible peptidoglycan precursors containing pentapeptides, blocking the following steps in cell wall formation. The D-Ala–D-Ala target residues are synthesized by the intracellular D-Ala:D-Ala ligase (Ddl). Acquired resistance to glycopeptides in enterococci by production of peptidoglycan precursors ending in the depsipeptide D-Ala–D-lactate (D-Lac) associated with elimination of the high-affinity D-Ala–D-Ala ending precursors is mediated by three classes of related operons, *vanA*, *vanB*, and *vanD* (14).

In VanB-type strains, two proteins are involved in the production of peptidoglycan precursors ending in D-Ala–D-Lac: $VanH_B$, a dehydrogenase that reduces pyruvate to D-Lac, and the VanB ligase that synthesizes D-Ala–D-Lac (18). The $VanX_B$ D,D-dipeptidase cleaves the D-Ala–D-Ala dipeptide synthesized by the host Ddl, and the $VanY_B$ D,D-carboxypeptidase contributes to vancomycin resistance by hydrolyzing the C-terminal D-Ala of the pentapeptide precursors when elimination by $VanX_B$ is not complete (2). The function of the additional VanW protein found in the *vanB* cluster is unknown. In *vanB* operons, expression of the $vanY_BWH_BBX_B$ resistance genes is controlled by the $vanR_BS_B$ genes, which encode a two-component regulatory system (10, 14). The regulatory and resistance genes are transcribed from promoters P_{RB} and P_{YB}, respectively, which are coordinately regulated (10, 11). In the presence of vancomycin, the membrane-bound $VanS_B$ sensor phosphorylates the cytoplasmic $VanR_B$ response regulator that enhances the transcriptional activation of both the $vanR_BS_B$ regulatory and $vanY_BWH_BBX_B$ resistance genes (11, 14).

VanB-type strains are resistant to various levels of vancomycin and susceptible to teicoplanin, which is not an inducer for these strains. Several VanB-type *Enterococcus faecium* and *Enterococcus faecalis* strains dependent on vancomycin for growth have been obtained in vitro (7), in animal models (5), and from humans (19, 29, 31) following prolonged treatment with this antibiotic. This phenotype is due to an inactive host Ddl following mutations in the *ddl* gene leading either to a truncated protein or to an amino acid substitution (7, 29). Consequently, these strains require the presence of vancomycin to induce the production of peptidoglycan precursors ending in D-Ala–D-Lac instead of D-Ala–D-Ala for growth. Two mechanisms leading to reversion to vancomycin resistance from vancomycin dependence have been described: (i) compensatory mutations in the *ddl* gene that restore the synthesis of D-Ala–D-Ala and the vancomycin inducible VanB phenotype (29) and (ii) mutations in the $vanS_B$ gene that lead to constitutive expression of the resistance pathway and resistance to both vancomycin and teicoplanin (7).

We have characterized three VanB-type *E. faecium* strains isolated from the same patient: the first strain, BM4659, was vancomycin resistant, the second, BM4660, was vancomycin dependent, and the third, BM4661, was a revertant resistant to

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
E. coli Top10	F^- *mcrA* Δ(*mrr hsdRMS mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
E. faecalis JH2-2	Fus^r Rif^r	22
E. faecium		
64/3	Fus^r Rif^r	30
BM4339	Vm^r Te^r [VanD type *ddl*(::5bp$_{37}$) $vanS_D$ ($C_{517}A$)]	8
BM4659	Vm^r Te^s (VanB type) Em^r Sp^r Sm^r Tc^r	This study
BM4660	Vm^d (VanB type) Em^r Sp^r Sm^r Tc^r	This study
BM4661	Vm^r Te^s (VanB-type revertant of strain BM4660) Em^r Sp^r Sm^r Tc^r	This study
BM4662	BM4339/pAT824 ($P_2ddl_{P175L}cat$) containing *ddl* gene of strain BM4660	This study
Plasmids		
pCR2.1	Ap^r Km^r, *oriR* from ColE1, *lacZ*α	Invitrogen
pAT79	*oriR* from pAMβ1, *oriR* from pUC, *oriT* from RK2, Sp^r *lacZ*α P_2cat	4
pAT824	1,135-bp SacI-XbaI PCR fragment (ddl_{P175L}) of strain BM4560 cloned in pAT79	This study
pIP846	Vm^r Em^r Sp^r Sm^r	This study

[a] Drug name abbreviations: Ap, ampicillin; Em, erythromycin; Fus, fusidic acid; Km, kanamycin; Rif, rifampin; Sm, streptomycin; Sp, spectinomycin; Te, teicoplanin; Vm, vancomycin. Superscripts: d, dependent; r, resistant; s, susceptible.

vancomycin and was susceptible to teicoplanin. Vancomycin-dependent strain BM4660 contained a mutation in the *ddl* gene leading to an amino acid substitution responsible for inactivation of the D-Ala:D-Ala ligase. However, vancomycin independence in strain BM4661 was not due either to a reversion in the *ddl* gene or to a mutation in the $vanS_B$ gene, two mechanisms that had been previously reported. In this clinical isolate, a single mutation was found in the $vanS_B$-$vanY_B$ intergenic region containing the P_{YB} resistance promoter region. This substitution was located in the transcription terminator of the $vanR_BS_B$ operon just upstream from the binding sites of the $VanR_B$ regulator. As a result, the $vanR_B$ and $vanS_B$ regulatory genes and the $vanY_BWH_BBX_B$ resistance genes were cotranscribed from the P_{RB} regulatory promoter due to transcriptional readthrough.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The origins and characteristics of the bacterial strains and plasmids used in this study are described in Table 1. *E. faecium* BM4659, BM4660, and BM4661 were isolated in 1999 from rectal swabs of a patient at the University Hospital Center of Montpellier, France. *Escherichia coli* Top10 (Invitrogen, Groningen, The Netherlands) was used as the host for recombinant plasmids. *E. faecalis* JH2-2 (22) and *E. faecium* 64/3 (30), resistant to fusidic acid and rifampin, were used as recipients in conjugation experiments. Ampicillin (100 μg/ml) was used as a selective agent for cloning PCR products into the pCR-2.1 vector (Invitrogen). Spectinomycin (60 μg/ml) was added to the culture medium to prevent loss of plasmids derived from pAT79 (4). Strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, MI) at 37°C. The MICs of glycopeptides were determined by the method of Steers et al. with 10^5 CFU per spot on BHI agar after 24 h of incubation at 37°C (27).

Recombinant DNA techniques. Plasmid DNA isolation, digestion with restriction endonucleases (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England), amplification of DNA by PCR with *Taq* or *Pfu* DNA polymerase (Stratagene, La Jolla, CA), ligation of DNA fragments with T4 DNA ligase (Amersham Pharmacia Biotech), and transformation of *E. coli* Top10 with recombinant plasmid DNA were performed by standard methods (6). Total DNA from enterococci was prepared as previously described (23).

Plasmid construction. The chromosomal *ddl* gene of vancomycin-dependent *E. faecium* BM4660, including its ribosome binding site (RBS), was amplified by PCR from total DNA with oligodeoxynucleotides 4147-1 and 4147-2 (8). Primers 4147-1 and 4147-2 contain, respectively, SacI and XbaI restriction sites that allow directional cloning of the *ddl* gene under the control of the constitutive P_2 promoter upstream from the *cat* reporter gene of the pAT79 shuttle vector (4). The 1,135-bp insert of the resulting pAT824($P_2ddl_{P175L}cat$) plasmid that contained the mutated *ddl* gene with the $P_{175}L$ mutation and its own RBS was resequenced.

Strain construction. Plasmid pAT824($P_2ddl_{P175L}cat$) DNA was introduced into *E. faecium* BM4339 (Table 1) by electrotransformation and selection on spectinomycin (120 μg/ml) to obtain *E. faecium* BM4662, and the transformants were screened for resistance to chloramphenicol. Plasmid DNA from chloramphenicol-resistant clones was digested with EcoRI plus HindIII, and the restriction profile was compared to that of pAT824($P_2ddl_{P175L}cat$) purified from *E. coli* Top10 to screen for DNA rearrangements.

DNA sequencing. Plasmid DNA was extracted with the commercial Wizard Plus Minipreps DNA purification system (Promega, Madison, WI), and the PCR fragments were purified with the microspins of the PCR purification kit (Qiagen). Plasmid DNA or PCR products were labeled with a dye-labeled dideoxynucleoside triphosphate Terminator Cycle Sequencing kit (Beckman Coulter UK Ltd.), and the samples were sequenced and analyzed with a CEQ 2000 automated sequencer (Beckman).

Computer analysis of sequence data. Determination of the degrees of identity and similarity to known proteins was carried out with BLASTN, BLASTX, BLASTP (1), and FASTA (26) from the Genetics Computer Group suite of programs. DNA Strider (version 1.4f3) was used for sequence analysis (16).

Contour-clamped homogeneous electric field gel electrophoresis. Genomic DNA embedded in agarose plugs was digested overnight at 27°C with 25 U of SmaI and separated on a 0.8% agarose gel with a CHEF-DRIII system (Bio-Rad Laboratories) under conditions described previously (15).

Plasmid-curing experiments. Vancomycin-resistant strain BM4659 was subcultured in BHI broth at 44°C, and after six passages, bacteria were plated on BHI agar. Isolated colonies were screened for susceptibility to vancomycin and erythromycin by replica plating. Plasmid loss was confirmed by disk agar diffusion and by agarose gel electrophoresis of the plasmid DNA.

Analysis of peptidoglycan precursors. Extraction and analysis by high-performance liquid chromatography of peptidoglycan precursors were performed as described previously (3). Enterococci were grown in BHI medium with or without vancomycin (4 μg/ml) to the mid-exponential phase (A_{600} = 1). Ramoplanin (3 μg/ml) was added to inhibit peptidoglycan synthesis, and incubation was continued for 15 min to cause accumulation of peptidoglycan precursors.

D,D-Dipeptidase ($VanX_B$) and D,D-carboxypeptidase ($VanY_B$) activities. The enzymatic activities in the supernatant and in the resuspended pellet fraction were assayed as described previously (3). Strains were grown until the optical density at 600 nm reached 0.7 in the absence or presence of vancomycin (8 μg/ml). The supernatant (S100) and resuspended pellet (C100) fractions were collected and assayed for D,D-peptidase (VanX or VanY) activities by measuring

TABLE 2. Oligonucleotide primers used as probes and in RT-PCR experiments

Primer[a]	Sequence (5′→3′)	Position[b]
VB78+	GCTGAGGCAGGATATCA	80–96
O7−	AGCAGCAAAAGGATTTC	495–479
VB26+	CGTATATCTTTGCGCGG	1130–1146
VB76−	CGATGGCAAGTCCTAAA	1918–1902
VB82+	CATCCCTGCAACTCATA	2357–2373
VB79−	CATCCAGCCATCTATAA	2829–2813
VB4+	TAACGCTGCGATAGAAG	5368–5384
VB2−	CTCTGCATCCAAGCACC	5657–5641
VB77−	CTGACAAGCTCCAGTAT	1529–1513
VB23−	AGAATTGTCGCCTCGGA	4034–4018
VB85−	GATGGATGCGGAAGATA	5531–5515
VB80−	CTGTGTTGCCTCCAATA	2348–2332

[a] +, sense; −, antisense.
[b] Nucleotide numbering begins at the transcriptional start site (+1) of the $vanR_B$ gene (10).

the D-Ala released from substrate hydrolysis (D-Ala–D-Ala at 6.56 mM or L-Ala–D-Glu–L-Lys–D-Ala–D-Ala at 5 mM) as described previously (3).

RNA techniques. (i) Extraction of total RNA. Total RNA was extracted with the commercially available Fast RNA Pro Blue kit (Qbiogene, Inc.) as follows. Enterococci were grown in BHI medium (15 ml) at 37°C to an optical density at 600 nm of 0.9, harvested by centrifugation, resuspended in 1 ml of RNA*pro* solution designed to inactivate cellular RNases efficiently during cell lysis to prevent RNA degradation, and disrupted with a FastPrep disintegrator (40 s at a setting of 6.0) by using a lysing matrix to release total cellular RNA, DNA, and proteins. After centrifugation for 5 min at 12,000 × *g*, supernatants were extracted with chloroform (0.3 ml), total RNA was precipitated by addition of 0.5 ml absolute ethanol, and the RNA pellets were resuspended in 100 μl water treated with diethyl pyrocarbonate (Sigma, Saint-Quentin Fallavier, France). RNA quality was assessed by agarose gel electrophoresis. RNA concentrations were determined by measuring A_{260}.

(ii) Northern analysis. Equal amounts of total RNA (20 μg) were separated under denaturing conditions in a 1.2% agarose formaldehyde-morpholinepropanesulfonic acid gel, stained with ethidium bromide, and blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech). The PCR products used as probes were obtained with total DNA from strain BM4659 as the template and the primers indicated in Table 2 and labeled with [α-^{32}P]dCTP (3,000 Ci nmol^{-1}; Amersham Pharmacia Biotech) by using the Megaprime DNA labeling kit (Amersham Pharmacia Biotech). Hybridization was carried out under stringent conditions, and washes were performed twice with 2× SSC (0.3 M NaCl plus 30 mM sodium citrate) and 0.5% sodium dodecyl sulfate at 60°C for 30 min.

(iii) RT-PCR experiments. Total RNA samples (50 μg) were digested with RNase-free DNase I (250 U) (Amersham Pharmacia Biotech) in a final volume of 1 ml for 10 min at 37°C. Samples were treated with phenol-chloroform and precipitated with ethanol. Reverse transcription (RT) was carried out (final volume, 20 μl) with 2 μg of purified RNA, 1× enzyme buffer (Superscript II kit; Invitrogen), 50 mM $MgCl_2$, 2 μg of bovine serum albumin (New England BioLabs, Inc., Beverly, MA) per ml, each of the four deoxyribonucleoside triphosphates at 1 mM (Amersham Pharmacia Biotech), 50 pmol of the VB77 or VB85 primer (Table 2), 20 U of RNase inhibitor (RNA guard; Amersham Pharmacia Biotech), and 200 U of Moloney murine leukemia virus modified reverse transcriptase. Samples were incubated for 30 min at 37°C, and the enzyme was inactivated at 95°C for 5 min. The DNA products were amplified by PCR in a final volume of 80 μl containing the previous 20-μl samples, 50 pmol each of the VB78-VB77, VB26-VB80, and VB82-VB23 pairs of primers (Table 2), 1× enzyme buffer (Amersham Pharmacia Biotech), and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The PCR (30 cycles) was performed in a Gene Amp PCR system 4800 (Perkin-Elmer Cetus, Norwalk, CT), and the products were separated on a 1% agarose gel, purified, and sequenced.

Nucleotide sequence accession numbers. The entire *vanB* cluster and the 1,154-bp fragment containing the *ddl* gene of revertant strain BM4661 were submitted to GenBank and assigned accession no. FJ767776 and FJ767775, respectively.

RESULTS AND DISCUSSION

Characterization of the clinical strains. In October 1999, a 66-year-old man was admitted to the cardiovascular unit of the University Hospital Center in Montpellier, France, and had aortic dissection surgery. Three days later, he was transferred to the intensive care unit for respiratory distress and renal insufficiency and prescribed cefepime and vancomycin. Three *E. faecium* strains, BM4659, BM4660, and BM4661, were isolated from rectal swabs. The first strain, BM4659, was resistant to vancomycin and susceptible to teicoplanin; BM4660, isolated 1 week later, was vancomycin dependent, whereas BM4661, isolated 20 days after the cessation of vancomycin therapy, was resistant to vancomycin but teicoplanin susceptible (Table 3). The glycopeptide resistance genotype and the species of the strains were determined by multiplex PCR (13), and the three strains were found to be VanB-type *E. faecium*.

The patterns obtained by pulsed-field gel electrophoresis after digestion of the total DNA of resistant strain BM4659 and dependent strain BM4660 by SmaI were indistinguishable, whereas revertant strain BM4661 had a single band shift (Fig. 1). The patterns of plasmid DNA extracted from the three clinical isolates after digestion with EcoRI, HindIII, and EcoRI-HindIII were indistinguishable, confirming that the strains were clonally related (Fig. 2 and data not shown). The location of the *vanB* gene cluster was determined by Southern hybridization with *vanB* and $vanS_B$ probes of plasmid DNA digested with EcoRI and HindIII (Fig. 2B). The *vanB* gene was assigned to a ca. 11-kb EcoRI fragment, and $vanS_B$ was assigned to a 2.77-kb EcoRI fragment (Fig. 2B) that also carried $vanR_B$, the $vanS_B$-Y_B intergenic region, and a portion of $vanY_B$ (data not shown) (20). The *vanB* and $vanS_B$ genes were assigned to the same 20-kb HindIII fragment (Fig. 2B). These results are consistent with the sequence of the *vanB* operon in

TABLE 3. MICs of glycopeptides against VanB-type enterococci and relative proportions of cytoplasmic peptidoglycan precursors

E. faecium strain	MIC (μg/ml) of[a]:		Inducing Vm concn (μg/ml)	% of peptidoglycan precursors[b]			
	Vm	Te		UDP-MurNAc-tripeptide	UDP-MurNAc-tetrapeptide	UDP-MurNAc-pentapeptide	UDP-MurNAc-pentadepsipeptide
BM4659	64	1	0	<1	<1	100	<1
			4	<1	35	23	42
BM4660	256	NA	4	39	5	12	44
BM4661	256	0.5	0	32	9	12	47
			4	28	6	10	56

[a] MICs were determined by the method of Steers et al. (27). Te, teicoplanin; Vm, vancomycin; NA, not applicable.
[b] Bacteria were grown without vancomycin to the mid-exponential phase, and peptidoglycan synthesis was inhibited by addition of ramoplanin (3 μg/ml) to the cultures and incubation for 15 min.

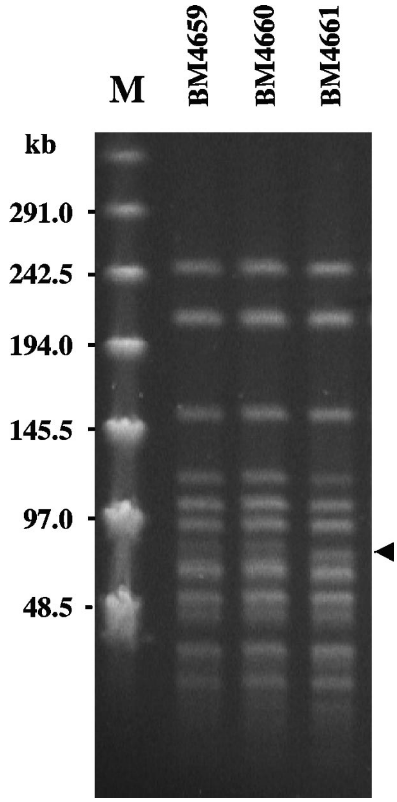

FIG. 1. Analysis of SmaI-digested genomic DNAs of the VanB-type clinical isolates by pulsed-field gel electrophoresis. Bacteriophage λ concatemers (lane M; New England BioLabs) were used as molecular size markers, and the sizes are indicated in kilobases at the left. The single band shift in revertant strain BM4661 in comparison with resistant strain BM4659 and dependent strain BM4660 is indicated by the arrowhead.

transposon Tn*1549* (Fig. 3). The *vanB* gene cluster was part of a plasmid of ca. 70 kb, designated pIP846, in resistant strain BM4659, dependent strain BM4660, and revertant strain BM4661. Resistance to erythromycin, due to an *ermB* gene, and to streptomycin and spectinomycin was also borne by plasmid pIP846. Loss of pIP846 from resistant strain BM4659 was associated with loss of resistance to vancomycin, erythromycin, streptomycin, and spectinomycin.

Repeated attempts to transfer vancomycin or erythromycin resistance from the clinical strains to *E. faecalis* JH2-2 or *E. faecium* 64/3 (Table 1) were unsuccessful.

Transition from vancomycin resistance to vancomycin dependence. Vancomycin-dependent strains require the antibiotic for growth. In the presence of vancomycin, production of the VanB D-Ala:D-Lac ligase is induced, which overcomes the defect in the synthesis of peptidoglycan precursors ending in D-Ala–D-Ala due to a lack of functional Ddl and thus allows growth of the host (7, 29). The sequence of the *ddl* gene and its upstream region in the three clinical isolates was thus determined. Comparative analysis revealed that the *ddl* sequence of resistant strain BM4659 was identical to that of prototype *E. faecium* BM4147 (17), whereas those of dependent strain BM4660 and revertant strain BM4661 had the same CCG-to-CTG mutation in codon 175, leading to a proline-to-leucine substitution. This $P_{175}L$ mutation was close to the lysine involved in the binding of D-Ala2 and is presumably responsible for a nonfunctional protein. The fact that the dependent strain BM4660 and revertant strain BM4661 *ddl* genes shared this mutation confirmed the clonal relationship of these strains.

VanD-type strain BM4339 (Table 1) is constitutively resistant to glycopeptides following a mutation in the $vanS_D$ gene

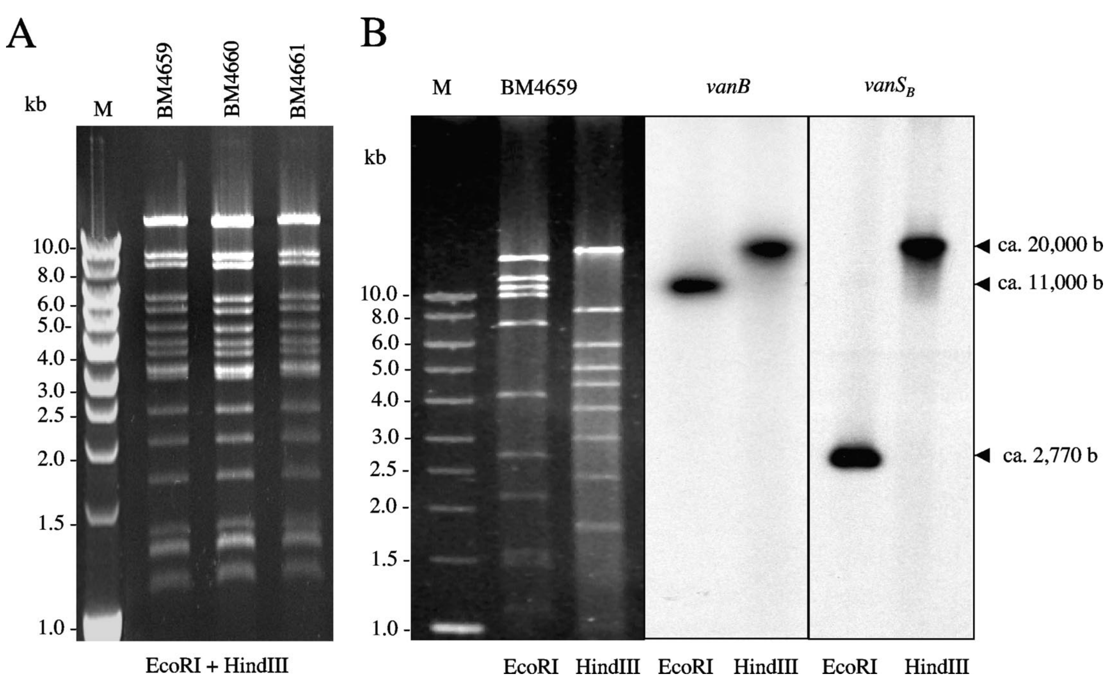

FIG. 2. Analysis by agarose gel electrophoresis of plasmid DNA from the VanB-type clinical isolates digested (A) with EcoRI and HindIII or from resistant strain BM4659 digested (B) with EcoRI or HindIII, followed by hybridization with *vanB* and $vanS_B$ probes. The sizes, in kilobases, of molecular size markers (lane M, 100-bp DNA ladder; Invitrogen) are indicated on the left. The sizes, in bases (b), of the fragments that hybridized are indicated on the right.

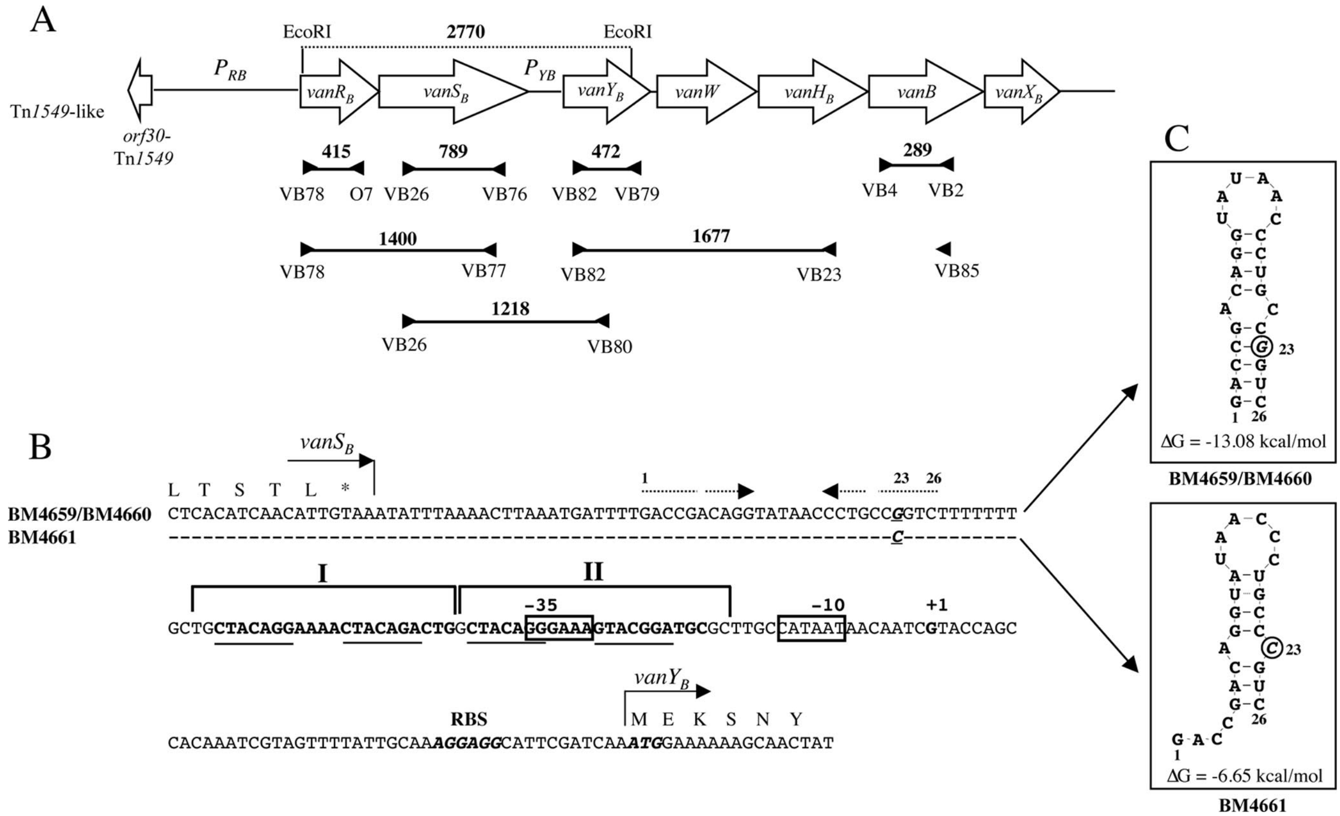

FIG. 3. Schematic representation of the *vanB* gene cluster of strain BM4659 and the sequence of the *vanS$_B$*-*vanY$_B$* intergenic region, including the P_{YB} resistance promoter. (A) Open arrows represent coding sequences and indicate the direction of transcription. The sizes, in base pairs, of the PCR products and the EcoRI fragments are indicated in bold. The probes used in Southern or Northern hybridizations and the oligodeoxynucleotides used for RT-PCR analysis are also shown. Arrowheads indicate the positions and orientations of the primers. The sizes of the PCR products are in bold. (B) The transcriptional start site (+1) is in bold, and the −35 and −10 sequences of P_{YB} are boxed. The translational start site (ATG) of *vanY$_B$* and the RBS are in boldface italics. Regions protected from DNase I cleavage by VanR$_B$ and phosphorylated VanR$_B$ are delineated by brackets I and II, respectively, and the conserved heptanucleotide direct repeats are underlined. The 21-bp consensus binding site of VanR$_B$ and phosphorylated VanR$_B$ is in boldface. The deduced amino acid sequences of the C-terminal portion of VanS$_B$ and the N-terminal portion of VanY$_B$ are shown above the nucleotide sequence and aligned with the first nucleotide of each codon. Horizontal arrows indicate regions of dyad symmetry. The G-to-C substitution in the inverted repetition of revertant strain BM4661 is in bold italics and underlined. The numbers indicate the bases in the transcription terminator. (C) Most stable conformation of the predicted RNA secondary structure of the transcription terminator in resistant strain BM4659, dependent strain BM4660 (top), and revertant strain BM4661 (bottom). The circle indicates the mutation in the transcription terminator of revertant strain BM4461. Δ*G* values were calculated with RNAfold (21).

and has an impaired Ddl enzyme (8, 15). Introduction of an intact *ddl* gene under the control of a constitutive promoter into this strain has been shown to restore the susceptibility of the host to glycopeptides (8). Decreased glycopeptide resistance is due to production of the heterologous Ddl enzyme since strain BM4339 possesses only very weak VanX$_D$ D,D-dipeptidase activity. To assess if the P$_{175}$L mutation was responsible for the impairment of Ddl, plasmid pAT824, containing the *ddl*$_{P175L}$ gene from strain BM4660 and its RBS cloned under the control of the constitutive P_2 promoter, was electrotransformed into strain BM4339 (Table 1). The resulting transformant, strain BM4662, remained vancomycin resistant (64 μg/ml), confirming that the D-Ala:D-Ala ligase of dependent strain BM4660 and revertant strain BM4661 was not functional. The vancomycin dependence of strain BM4660 can be accounted for by an impaired Ddl protein, whereas strain BM4661, which is resistant to vancomycin even in the absence of this antibiotic and was isolated later, must have suffered a compensatory mutational event. As examples for this hypothesis, VanD-type strains, despite an inactive D-Ala:D-Ala ligase, are able to grow in the absence of glycopeptide since the *vanD* clusters are expressed constitutively due to mutations either in the VanS$_D$ sensor or in the VanR$_D$ regulator (12, 15). In a VanB-type strain, increased glycopeptide resistance was shown to be due to the combination of a frameshift mutation resulting in a nonfunctional Ddl ligase and the constitutive synthesis of pentadepsipeptide precursors secondary to loss of VanS$_B$ phosphatase activity (11).

Characterization of the peptidoglycan precursors. The nature and relative amounts of the cytoplasmic precursors synthesized by the three strains were determined. In the absence of vancomycin, resistant strain BM4659 accumulated exclusively UDP-MurNAc-pentapeptide, whereas in the presence of the drug, UDP-MurNAc-pentadepsipeptide (42%) was the main precursor produced but UDP-MurNAc-tetrapeptide (35%) and UDP-MurNAc-pentapeptide (23%) were also present (Table 3). The presence of UDP-MurNAc-pentapeptide could account for the moderate level of resistance to vancomycin. The tetrapeptide is likely to result from the removal of D-alanine from UDP-MurNAc-pentapeptide by $VanY_B$. This D,D-carboxypeptidase acts if the elimination of D-Ala–D-Ala by $VanX_B$ is incomplete (2). Vancomycin-dependent strain BM4660 synthesized mainly UDP-MurNAc-pentadepsipeptide (44%), whereas large amounts of UDP-MurNAc-tripeptide (39%) and small amounts of pentapeptide (12%) and tetrapeptide (5%) were present (Table 3). In vancomycin revertant strain BM4661, synthesis of peptidoglycan precursors was similar to that in dependent strain BM4660 and no differences were observed in cells grown in the absence or presence of vancomycin, suggesting constitutive expression of the resistance genes. The presence of tripeptide in large quantity suggests that the VanB ligase may not be sufficiently active to synthesize D-Ala–D-Lac as rapidly as tripeptide is produced. Since the D-Ala:D-Ala ligase of dependent strain BM4660 and revertant strain BM4661 is inactive, little, if any, UDP-MurNAc-pentapeptide should be present; therefore, the small amount of pentapeptide could have been synthesized by the VanB ligase, as already shown (24).

Reversion from vancomycin dependence to vancomycin resistance. Analysis of the peptidoglycan precursors and of the *ddl* sequence supports the notion that the phenotype of revertant strain BM4661 is due to constitutive expression of resistance. As mentioned above, constitutive glycopeptide resistance in VanB- and VanD-type enterococci is due to mutations in the $vanS_B$ gene or in the $vanS_D$ or $vanR_D$ gene, respectively (7, 12, 15). Mutations in $VanS_B$ can lead to permanent phosphorylation of $VanR_B$ due to loss of the phosphatase activity of the sensor and thus to constitutive expression of resistance (11). However, comparison of the sequences of $vanR_B$ and $vanS_B$ from resistant strain BM4659, dependent strain BM4660, and revertant strain BM4661 revealed no mutations. PCR mapping with primers complementary to the *vanB* operon of *E. faecalis* BM4524 (11) gave fragments of the expected size, indicating that in the three strains, all of the genes constituting the *vanB* operon were present with the same organization (Fig. 3A) and that no large insertions or deletions occurred in the noncoding regions. The entire sequence of the *vanB* operon, including the P_{RB} and P_{YB} promoter regions, was determined and found to be identical in the three strains except for a single nucleotide mutation (G to C) located in the $vanS_B$-$vanY_B$ intergenic region of revertant strain BM4661. This transversion was located upstream from the binding sites of the $VanR_B$ regulator in the P_{YB} resistance promoter region, in the region of dyad symmetry that corresponds to the putative transcription terminator of the $vanR_BS_B$ operon (Fig. 3B). The deduced amino acid sequences of $VanR_B$, $VanS_B$, $VanY_B$, VanW, $VanH_B$, VanB, and $VanX_B$ were 99.1, 100, 100, 96, 98.5, 99.4, and 96.5% identical, respectively, to those of the corresponding deduced proteins of transposon Tn*1549* (20). Based on sequence differences, the *vanB* gene clusters have been divided into three subtypes, *vanB-1*, *-2*, and *-3* (25), and the strains studied belong to the *vanB-2* genotype.

Structure of the transcription terminator of the $vanR_BS_B$ operon. In prokaryotes, genes belonging to an operon are transcribed in a single mRNA molecule. Transcription starts at the binding site of RNA polymerase in the promoter region and continues until it reaches a transcriptional terminator. An intrinsic transcriptional terminator is generally present immediately downstream of stop codons and is characterized by the presence of a G/C-rich palindromic region, followed by multiple T residues, yielding an RNA with a stem-loop structure, followed by multiple U residues (28). This nascent RNA stem-loop secondary structure causes pausing of the polymerase and weakens the interaction of the polymerase with the nascent RNA and template DNA facilitated by the weak hybrid formed by the dA-rU base pairing of the T trail. Inverted repeats, able to form a hairpin structure for termination of transcription and with all of the expected characteristics, were identified downstream of $vanS_B$ of resistant strain BM4659 and dependent strain BM4660 (Fig. 3). The single difference in the sequences of the three *vanB* operons was in the putative terminator of the $vanR_BS_B$ operon of revertant strain BM4661, suggesting that reversion in this strain could be due to overcoming of transcription termination. The strength of the two versions of transcriptional terminators was calculated with RNAfold (21) and Mfold (32), and the mutation in the stem of the $vanR_BS_B$ terminator entailed a free energy decrease from −13.08 to −6.65 kcal/mol (Fig. 3C). The predicted ΔG for the terminator of resistant strain BM4659 and dependent strain BM4660 was similar to those of canonical terminator sequences of *Bacillus subtilis* (−14 kcal/mol) and *E. coli* (−16 kcal/mol) (9), whereas that of revertant strain BM4661 was approximately half. We have shown that the RNA polymerase was able to interact with the P_{RB} promoter of the regulatory genes in the absence of $VanR_B$ but with the P_{YB} promoter of the resistance genes only in the presence of the regulator (10). Moreover, low levels of transcripts initiated at P_{RB}, but not at P_{YB}, were detected in in vitro runoff transcription experiments in the absence of $VanR_B$ (10). These data account for the low-level production of the two-component regulatory system and for the lack of expression of the resistance genes in the absence of induction.

Taken together, these results indicate that destabilization of transcription termination of the $vanR_BS_B$ operon allowed readthrough, leading to the coexpression of $vanY_B$, *vanW*, $vanH_B$, *vanB*, and $vanX_B$. This represents a new mechanism for reversion from vancomycin dependence to vancomycin resistance that had not yet been observed.

D,D-Peptidase activities. The D,D-dipeptidase activity was measured in the $100{,}000 \times g$ cytosolic fraction of bacterial crude extracts following growth in the absence or presence of 8 μg/ml vancomycin. As expected, in resistant strain BM4659, D,D-dipeptidase synthesis was inducible by vancomycin only (Table 4). In the presence of vancomycin, $VanX_B$ activity in vancomycin-dependent strain BM4660 and revertant strain BM4661 was lower than in strain BM4659. Since strains BM4660 and BM4661 do not produce D-Ala–D-Ala-ending peptidoglycan precursors following impairment of the chromosomal *ddl* gene, D,D-dipeptidase activity is virtually unnecessary for glycopeptide resistance in these strains. However, in revertant strain BM4661, and opposed to strain BM4659, substantial D,D-dipeptidase activity was detected even in the absence of vancomycin and was increased in the presence of the drug (Table 4). In the absence of glycopeptides, only the $vanR_BS_B$ regulatory genes are expressed and at a low level from P_{RB}, whereas under inducing conditions, the $VanR_B$ phosphorylated regulator activates both the P_{RB} regulatory and P_{YB} resistance promoters, resulting in highly increased transcription of the regulatory and resistance genes (10, 11). Thus, the D,D-

TABLE 4. D,D-Peptidase activities in extracts from *E. faecium* BM4659 and its derivatives

Strain	Inducing vancomycin concn (μg/ml)	Mean D,D-dipeptidase activity ± SD (nmol min^{-1} mg^{-1})[a]	Mean D,D-carboxypeptidase activity ± SD (nmol $min^{-1}mg^{-1}$) in[b]:	
			Membrane fraction	Cytoplasmic fraction
BM4659	0	1.5 (±0.4)	7 (±0.5)	2.3 (±0.4)
	8	255 (±33)	115 (±25)	13 (±4)
BM4660	8	99 (±12)	30 (±11)	5.3 (±0.6)
BM4661	0	30 (±2)	8 (±0.5)	1.3 (±0.2)
	8	128 (±23)	27 (±2)	4.3 (±0)

[a] The activity was measured in the 100,000 × *g* supernatant of lysed bacteria. The results shown were obtained from a minimum of three independent extracts.
[b] The activity was measured in the supernatant or the resuspended pellet fraction after centrifugation of lysed bacteria at 100,000 × *g* for 45 min.

dipeptidase activity observed in revertant strain BM4661 in the absence of vancomycin is consistent with transcriptional readthrough of the resistance genes from the P_{RB} regulatory promoter.

The D,D-carboxypeptidase specific activity in cytoplasmic fractions of the three clinical isolates was significantly lower than that in membrane extracts, consistent with the fact that $VanY_B$ is a membrane-bound protein. Results similar to those obtained with $VanX_B$ were obtained with membrane preparations of strain BM4659, expression of the structural gene being inducible by vancomycin only and the high levels of $VanY_B$ produced accounted for the elevated amounts of tetrapeptides (Table 4). Since strains BM4660 and BM4661 produce a very small amount of pentapeptide (Table 3), no D,D-carboxypeptidase activity is required for glycopeptide resistance (Table 4).

Transcriptional analysis of the *vanB* gene clusters. Total RNAs extracted from resistant strain BM4659 and revertant strain BM4661 left uninduced or induced by vancomycin were analyzed by Northern hybridization (Fig. 4A) with probes internal to $vanR_B$, $vanS_B$, and $vanY_B$ (Fig. 3A).

In strain BM4659, a transcript of ca. 2,000 nucleotides that hybridized with the $vanR_B$ and $vanS_B$ probes was obtained with or without induction, albeit in much larger amounts in the presence of vancomycin. This observation is consistent with the fact that, in order to sense the presence of vancomycin in the medium, VanB-type strains need to synthesize small amounts of $VanR_B$ and $VanS_B$ molecules in the absence of inducer (10). In contrast, a transcript of ca. 4,300 nucleotides that hybridized with the $vanY_B$ probe was

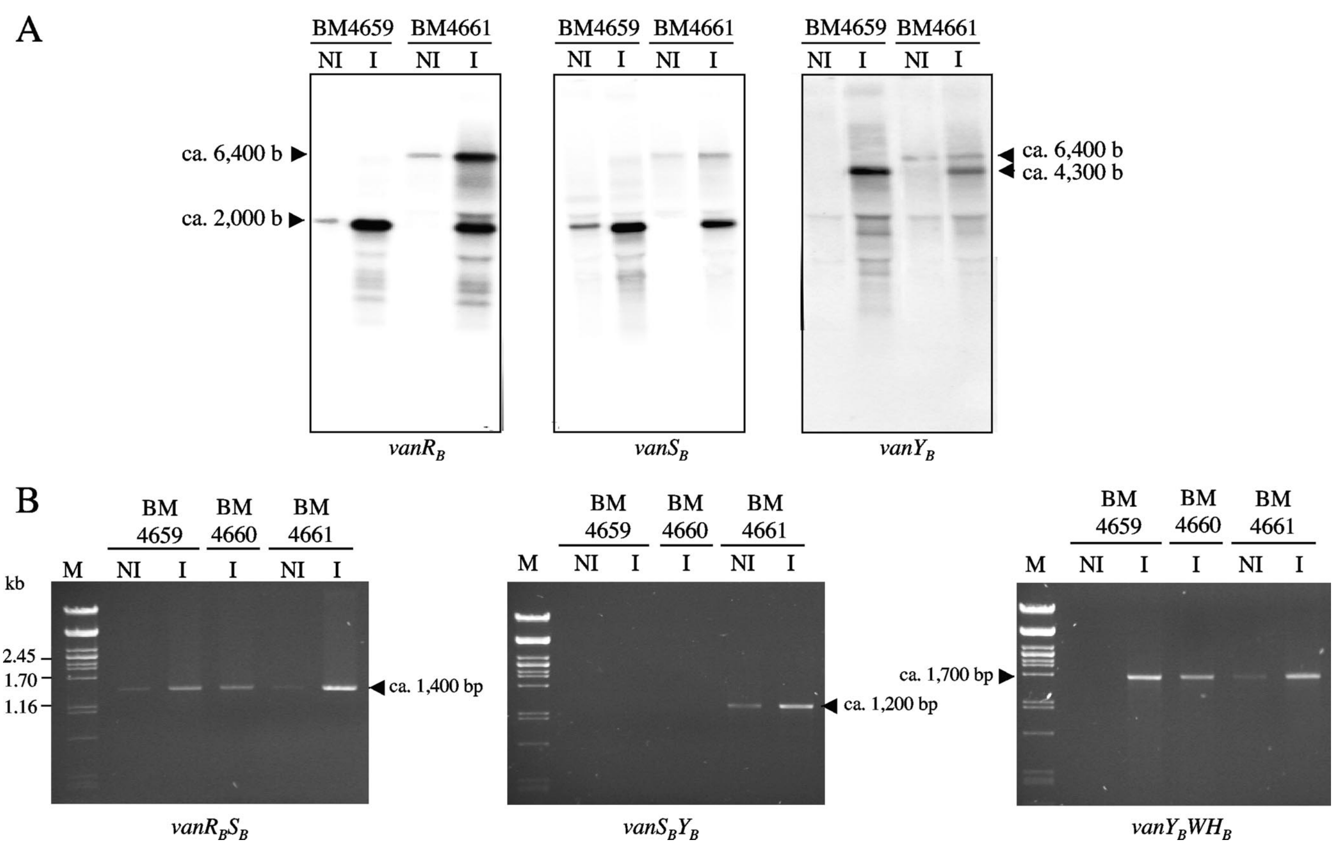

FIG. 4. Transcription analysis of the *vanB* gene cluster by (A) Northern hybridization and (B) RT. (A) Total RNAs from resistant strain BM4659 and revertant strain BM4661 left uninduced (NI) or induced (I) with vancomycin were hybridized successively with $vanR_B$, $vanS_B$, and $vanY_B$ probes. The size of the transcript indicated on the side was determined according to RNA molecular weight marker I (Boehringer) (not shown). (B) Transcription analysis of the $vanR_B$-$vanS_B$, $vanS_B$-$vanY_B$, and $vanY_BWH_B$ genes of resistant strain BM4659, dependent strain BM4660, and revertant strain BM4661. Agarose gel electrophoresis of RT-PCR products from noninduced (NI) and induced (I) strains with primer pairs VB78-VB77, VB26-VB80, and VB82-VB23, respectively (Fig. 3B and Table 2). Lanes M, bacteriophage λ DNA digested with PstI and used as size markers. The sizes of the PCR products are in base pairs.

obtained only in the presence of vancomycin, indicating that the $vanY_BWH_BBX_B$ resistance genes are cotranscribed in an inducible manner only.

In revertant strain BM4661, the transcripts of ca. 2,000 and 4,300 nucleotides were observed only in the presence of vancomycin whereas a larger transcript of ca. 6,400 nucleotides that hybridized with the $vanR_B$, $vanS_B$, and $vanY_B$ probes was obtained with or without induction (Fig. 4A). These results indicate that in strain BM4661 the $vanY_BWH_BBX_B$ resistance genes are cotranscribed with the $vanR_BS_B$ regulatory genes under the control of the P_{RB} promoter even in the absence of vancomycin.

The Northern hybridization results were confirmed by RT experiments (Fig. 4A and B). Regulation of transcription of $vanR_BS_B$ was studied with purified total RNA from induced and uninduced strains BM4659, BM4660, and BM4661 and primer VB77 (internal to $vanS_B$). The cDNA was amplified with primers VB78 and VB77 (internal to $vanR_B$ and $vanS_B$, respectively) (Fig. 3A and Table 2). After separation on agarose gel, a PCR product with the expected size of ca. 1.4 kb was obtained from cells grown with or without induction, although in much greater amounts after growth in the presence of vancomycin. Taken together, these results confirm that the $vanR_B$ and $vanS_B$ genes are cotranscribed and that the corresponding mRNA starts upstream of $vanR_B$. Transcription analysis of $vanY_B$, *vanW*, $vanH_B$, *vanB*, and $vanX_B$ was performed with total RNA from the three strains grown in the presence or absence of vancomycin with primer VB85 (internal to *vanB*) (Fig. 3A and Table 2). The cDNA was amplified with primers VB82 and VB23 (Table 2) (internal to $vanY_B$ and $vanH_B$, respectively). A PCR product with the expected size of 1.7 kb was detected in resistant strain BM4659 and dependent strain BM4660 only in the presence of vancomycin, whereas in revertant strain BM4661 this product was present also in the absence of vancomycin, although in much lower amounts than after growth under inducing conditions (Fig. 4B). The amplification products were sequenced, and no DNA rearrangements were found. These results confirm that the resistance genes are cotranscribed in an inducible manner in strains BM4659 and BM4660. In contrast, and only in revertant strain BM4661, a PCR product of 1.2 kb was obtained when RT was performed with primer VB80 (internal to $vanY_B$) and the corresponding cDNA amplified with primers VB26 and VB80 (internal to $vanS_B$ and $vanY_B$, respectively). This product includes the intergenic region with the P_{YB} resistance promoter and the transcriptional terminator (Fig. 3 and 4B), and its presence confirms the cotranscription of the $vanR_B$ $S_BY_BWH_BBX_B$ genes in revertant strain BM4661.

This study illustrates a third mechanism of reversion from vancomycin dependence to resistance in a VanB-type clinical isolate due to a mutation in the transcriptional terminator of the $vanR_BS_B$ regulatory genes resulting in transcriptional readthrough of the resistance genes from the regulatory promoter in the absence of vancomycin.

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